3079-Pos Board B184

The Cardiac L-Type Ca^{2+} Channel is Downregulated by Ischemic and Pharmacological Preconditioning

Jorge A. Sanchez, German González, Daniel Zaldívar, Elba D. Carrillo, Maria C. García.

Ischemic preconditioning (IP) is a phenomenon in which briefs periods of ischemia activate endogenous mechanisms that protect cardiomyocytes from subsequent ischemia. Pharmacological Preconditioning (PPC) with mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) openers such as diazoxide mimics IP. However, changes in Ca^{2+} homeostasis during IP and PPC, particularly in Ca^{2+} channel activity, are poorly understood. We investigated the effects of IP and PPC on cardiac L-type Ca^{2+} channels.

IP was achieved by four cycles of ischemia-reperfusion in isolated hearts**.** PPC was induced in isolated hearts and in dissociated cardiomyocytes from adult rats by preincubation withdiazoxide. We measured reactive oxygen species (ROS) production and Ca^{2+} signals associated with action potentials usingfluorescent probes. L-type currents were measured with the whole-cell patch-clamp technique. Levels of the $alpha_{1c}$ subunit of L-type channelsin the cellular membrane were measured by Western blot.

IP produced a 20% reduction in $alpha_{1c}$ subunit levels. PPC was accompanied by a 50% reduction in $alpha_{1c}$ subunit levels, and by a reversible fall in L-type current amplitude and Ca^{2+}transients. These effects were prevented by the ROS scavenger N-acetyl-L-cysteine (NAC) or by the mitoK_{ATP} channel blocker5-hydroxydecanoate (5-HD). PPC significantly reduced infarct size, an effect blocked by NAC and 5-HD. Nifedipine alsoconferred protection against infarction when applied during the reperfusion period. Downregulation of the $alpha_{1c}$ subunit andCa^{2+} channel function were prevented in part by the protease inhibitor leupeptin.

We conclude that IP andPPC downregulate the $alpha_{1c}$ subunit by a proteolytic process in which ROS are involved. This in turn leads to a reduced Ca^{2+} influx and attenuates Ca^{2+} overload during reperfusion.

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Expression Pattern of L-Type Calcium Channel Subunits in Human and Murine Atherosclerosis

Ann Kristin Boehnke, Margarete Odenthal, Michael Gawenda, Jan Matthes, Peter Hein, Stefan Herzig.

Cardiovascular L-type Ca^{2+}-channels (LTCC) are heteromeric protein complexes consisting of a pore-forming $Ca_v1.2$ (α_{1C}) and several auxiliary subunits. Major determinants of channel function include the composition of the heteromeric complex, but also subunit transcript regulation by splicing. This is of particular importance since splicing patterns may change under pathophysiological conditions, such as atherosclerosis. In human atherosclerosis, arterial smooth muscle $Ca_v1.2$ isoforms containing exon 21 were reported to be replaced by a single isoform containing the alternative exon 22. We have recently shown that this splice shift has major effects on LTCC: co-expressing a β_3-subunit together with α_{1C70} (containing exon 21) led to markedly reduced current density compared to α_{1C77} (containing exon 22). Co-expression of a β_2-subunit with either α_{1C70} or α_{1C77} had the opposite effect.

In the present work we sought to determine the expression pattern of LTCC subunits in both murine and human atherosclerotic arterial tissue. Murine aorta was isolated from wild-type and atherosclerotic apoE-knockout mice. Samples of human atherosclerotic arteries were obtained from patients undergoing arterial bypass surgery, and vascular smooth muscle cells were isolated by laser microdissection. qRT-PCR was used to determine LTCC subunit expression.

In murine aortic tissue we were able to detect exon 21- as well as exon 22-containing $Ca_v1.2$ subunit mRNA. Furthermore, we detected β_1-, β_2- and β_3-subunit mRNA with expression of β_1-subunits being significantly reduced in atherosclerotic aortic tissue compared to non-atherosclerotic tissue. In human atherosclerotic tissue samples, we found mRNA expression of all four β-subunits, $\beta_{1\text{-}4}$, with a trend towards β_2-subunit mRNA being more abundant than that of other β-subunit isoforms.

Given the functional impact of LTCC composition, our data support the idea that altered expression profiles of b-subunits might affect channel function in atherosclerosis.

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Novel Blockers of T-Type Calcium Channels Modify Gating

Pamela Bergson, Victor N. Uebele, John J. Renger, Dorothy A. Hanck.

Physiological and biophysical studies of T-type calcium channels, a subclass of voltage gated ion channels with a characteristically low voltage threshold for activation, have been hampered by a lack of selective antagonists, and no specific blockers of T-type calcium channels are currently clinically available. Several compounds have recently been identified that block calcium entry into cells expressing T-type calcium channels (Uebele et al, Cell Biochem Biophys 55:81). These compounds include an amide (TTA-A2) and a quinazolinone (TTA-Q4). We used whole cell patch clamp to examine block by these two experimental drugs in HEK 293 cells heterogeneously expressing $Ca_V3.1$. When holding potential was −110 mV, the TTAs blocked resting T-type channels with sub-micromolar affinity (ED_{50}=890nM for TTA-A2 and 560nM for TTA-Q4). Block was voltage dependent, and the affinity rose by approximately 5-fold when tested at −20 mV (ED_{50}=200nM for TTA-A2 and 130nM for TTA-Q4). Although the extent of block was voltage dependent, the kinetics were not. Neither the rate of development of block (τ=100ms for TTA-A2 and 178ms for TTA-Q4) nor the recovery from block (τ=1.2 sec for TTA-A2 and 4 sec for TTA-Q4) changed with voltage, consistent with a guarded receptor model. An examination of gating current revealed that both TTA-A2 and TTA-Q4 inhibited the movement of gating charge at depolarized potentials, without shifting the half-point of activation. These findings suggest that TTA-A2 and TTA-Q4 inhibit T-type current by restricting the movement of one or more voltage sensors, which may prevent the opening of the channel.

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Amiloride Docking to T-Type Calcium Channels

Manuel Rivera, Osbaldo López-Charcas, Juan C. Gomora.

Amiloride and its analogues are small molecules frequently used as potassium-sparing diuretics. These drugs have been utilized for decades in the treatment of diseases like hypertension. They are known for interacting with the sodium/hydrogen antiporter, sodium/calcium exchanger, and mainly with the epithelial sodium channel (ENaC). However, amiloride has also served as a pharmacological tool for the study of T-type calcium channels. The structural basis for these interactions has not been elucidated as crystal structures of these proteins are not known yet. Nevertheless, a possible interpretation of the amiloride blocking effects is that these proteins share common structural homologies. In this work we examined the interaction of amiloride with T-type voltage-gated calcium channels (Ca_V3), whose protein structure was available by computational modeling approach. Using molecular docking software, amiloride and related molecules were docked to Ca_V3 channels model structures in order to unveil potential interaction sites. The resulting predictions were experimentally validated by assessing the effect of amiloride on Ca_V3 channels expressed in HEK-293 cells, with the use of the whole-cell patch-clamp technique. The results showed differential sensitivity of Ca_V3 family members to amiloride, being $Ca_V3.2$ the most sensitive to the diuretic. The difference with $Ca_V3.1$ and $Ca_V3.3$ was more than an order of magnitude. These results offer the possibility to propose a model of electrostatic interactions of the putative sites with members of Ca_V3 family, which interact with drugs that share a similar structure with amiloride.

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Interaction of Diltiazem with an Intracellularly Accessible Binding Site on $Ca_V1.2$

Stanislav Beyl, Waheed Shabbir, Eugen Timin, Denise Schellmann, Thomas Erker, Annette Hohaus, Gregory H. Hockerman, Steffen Hering.

Diltiazem inhibits $Ca_V1.2$ channels and is widely used in clinical practiceto treat cardiovascular diseases. Binding determinants for diltiazem are located on segments IIIS6, IVS6 and the selectivity filter of the pore forming α_1 subunit of $Ca_V1.2$. Here we elucidate the access path of Diltiazem to its binding site making use of membrane impermeable quaternary derivative qDil and mutant α_1 subunits. qDil was synthesized and applied to the intracellular side (via the patch pipette) or to the extracellular side of the membrane (by bath perfusion). qDil inhibits $Ca_V1.2$ when applied to the intracellular side of the membrane in a use-dependent manner ($59 \pm 4\%$ at 300 μM) and induced only a low level of tonic (non use-dependent) block ($16 \pm 2\%$ at 300 μM) when applied to the extracellular side of the membrane. Mutations in IIIS6 and IVS6 reduced sensitivity to intracellularly applied qDil.Our study demonstrates intracellular access of quaternary diltiazem and its interaction with previously identified determinants of BTZ binding pocket. Recovery from block by diltiazem was found to be voltage independent, which is in contrast with the pronounced voltage dependent recovery from block by phenylalkylamines.